Determination of 2,3-*p*-Dioxanedithiol *S,S*-bis(*O,O*-diethyl Phosphorodithioate)

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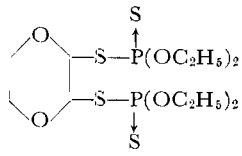
Development of a new organophosphorus pesticide, 2,3-*p*-dioxanedithiol *S,S*-bis(O,O-diethyl phosphorodithioate) required both assay and residue methods. A cleavage reaction peculiar to thioacetals is employed to convert this compound, quantitatively, to glyoxal. This dicarbonyl compound is determined either gravimetrically or colorimetrically as the 2,4-dinitrophenylosazone. Chromatographic procedures are simplified and speeded by use of a compact apparatus, which provides support, vacuum, and pressure for a bank of various chromatographic tubes. The method has been applied successfully to assay analyses and to a variety of crops for residue determinations.

THE INCREASING USE of organic compounds as agricultural chemicals demands more specific analytical methods for individual compounds, which are not based upon measurements of elements, groups, or properties that are present in other commonly used materials. An accurate knowledge of crop residues is essential to the estimation of agricultural chemical health hazards and is an important factor in establishing residue tolerances.

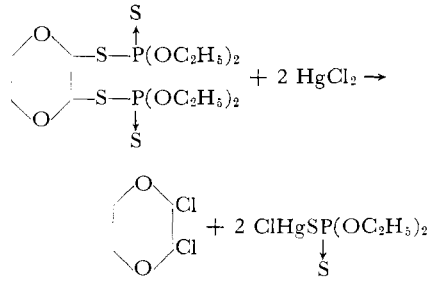
2,3-*p*-Dioxanedithiol *S,S*-bis(*O,O*-diethyl phosphorodithioate), Delnav (formerly Hercules AC-528, Hercules Powder Co.), an organic thiophosphate now in commercial development, has the following structure:



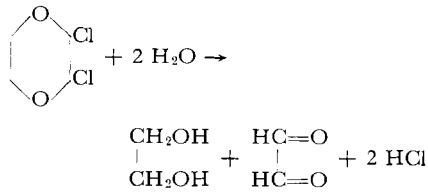
The method developed is based upon reactions involving the specificity of the dioxane portion of the molecule.

The structure of Delnav indicates that reactions of thioacetals would be of analytical use. Thioacetals are subject to cleavage of the carbon-sulfur bond when treated with mercuric chloride. Emil Fischer (*3*) first established this in some of his sugar studies, and the technique has been used ever since (*13*, *16*, *17*). Holmberg (*5*, *6*) made use of this cleavage technique in his work on the analysis of mercaptalacetic and mercaptoleacetic acids.

Cleavage of carbon-sulfur bonds in the case of Delnav yields 2,3-dichloro-*p*-dioxane as one of the reaction products:



The hydrolysis of 2,3-dichloro-*p*-dioxane has been investigated (*2*, *15*); it yields glyoxal and ethylene glycol according to the following equation:



The cleavage and hydrolysis reactions given above are the basis for the method described in this paper. The glyoxal is converted continuously as it is formed to the 2,4-dinitrophenylosazone by the use of a reagent containing 2,4-dinitrophenylhydrazine perchlorate, mercuric chloride, and water. This derivative was chosen because of its ease of preparation, high molecular weight, and great insolubility in most solvents. In addition, when made basic, the derivative yields an intense blue color that is well suited for spectrophotometric measurements. Thus, Delnav can be converted to glyoxal 2,4-dinitrophenylosazone, and this compound may be measured gravimetrically for assay purposes or colorimetrically for residue analysis.

Interferences encountered in the gravimetric determination of Delnav are of two types:

A. Materials coprecipitating with the glyoxal derivative and those occluded by or adsorbed on the precipitate.

B. Glyoxal precursors other than Delnav which are present in the technical product. Reagents for the elimination of Type A materials have been devised and Type B interferences can be eliminated by a simple treatment with an adsorbent such as alumina, prior to the cleavage-hydrolysis reaction.

In the colorimetric modification of the method, the chief interferences are Type B materials and any colored compounds such as excess reagent or hydrazones and osazones formed from reagent impurities and crop extractives. Type B interferences are handled as with the gravimetric method, and extraneous colors are circumvented by purifying the glyoxal derivative prior to its colorimetric determination, using adsorption chromatography.

Analytical Procedures

Gravimetric Cleavage—Hydrolysis Procedure Severe interference can result from the presence in the sample of glyoxal precursors other than Delnav. In the analysis of technical Delnav and its formulations, alumina is recommended for the removal of these interfering compounds. Details concerning such a preliminary cleanup are given in the colorimetric procedure under Preparation of Standard Curve.

Reagents and Apparatus. Mercuric chloride, reagent grade, 0.2*M*, in alcohol. Dissolve 27 grams of reagent grade

mercuric chloride in ethyl alcohol and dilute to 500 ml.

2,4-Dinitrophenylhydrazine, 0.08*M*, in perchloric acid. Dilute 100 ml. of 70%, reagent grade, perchloric acid with 100 ml. of water. Add slowly, with stirring, 4.8 grams of 2,4-dinitrophenylhydrazine. Dilute with an additional 100 ml. of water.

Potassium iodide–hydrochloric acid reagent. Dissolve 15 grams of reagent grade potassium iodide in a mixture of 10 ml. of concentrated hydrochloric acid and 85 ml. of water.

N,*N*-Dimethylformamide, (DMF) E. I. du Pont de Nemours & Co., Inc., technical grade.

Filtering crucibles, fritted glass, fine porosity, Gooch high-type, 30-ml. capacity, or Selas No. 3010.

Procedure. Weigh a sample that will contain 70 to 140 mg. of Delnav into a 50-ml. Erlenmeyer flask, equipped with ground-glass joints and matching reflux condensers. Add 10 ml. of mercuric chloride solution and 15 ml. of dinitrophenylhydrazine reagent. Reflux for 15 minutes. Carborundum boiling stones are sufficient to prevent bumping if hot plate temperature is just sufficient to keep the solution boiling. Excessive heat may cause severe bumping.

Cool to room temperature, and filter through a filtering crucible. Wash the precipitate with two 20-ml. portions of aqueous ethyl alcohol (1 to 1) and follow with two 10-ml. portions of 95% ethyl alcohol. Wash with at least two 20-ml. portions of potassium iodide–hydrochloric acid solution to dissolve any precipitated mercury compounds. Because of the flocculent nature of the osazone precipitate, it must be rubbed with a rubber policeman to ensure adequate contact between the potassium iodide–hydrochloric acid reagent and occluded or adsorbed mercury compounds. Wash with two 20-ml. portions of aqueous ethyl alcohol, once with 10 ml. of 95% ethyl alcohol, and then with two 15-ml. portions of reagent grade carbon disulfide. Wash with 10 ml. of 95% ethyl alcohol, and dry for 15 minutes in a forced-draft oven at 105° C. Cool for at least 30 minutes and weigh. Dissolve the precipitate under the hood in hot dimethylformamide, using a suction flask to remove the solution, and finally wash with 95% ethyl alcohol. Dry and weigh the crucible as before and calculate per cent Delnav as follows:

$$\frac{\text{Weight of crucible + precipitate − weight of crucible after } N,N\text{-dimethylformamide}}{\text{sample weight}} \times \frac{456}{418} \times 100 = \% \text{ Delnav}$$

where molecular weight of Delnav is 456 and molecular weight of glyoxal 2,4-dinitrophenylosazone is 418.

Colorimetric Cleavage—Hydrolysis Procedure

Reagents and Apparatus. Mercuric chloride, 0.2*M* in 95% ethyl alcohol. Dissolve 2.7 grams of reagent grade mercuric chloride in 50 ml. of 95% ethyl alcohol that has been purified by refluxing with 2,4-dinitrophenylhydrazine and sulfuric acid and by distilling.

2,4-Dinitrophenylhydrazine, 0.08*M* in 32% perchloric acid. Dilute 33 ml. of 70% reagent grade perchloric acid with 33 ml. of water. Dissolve 1.6 grams of 2,4-dinitrophenylhydrazine in this and add 33 ml. of water.

Acetone. Hercules Powder Co. acetone is satisfactory. Distillation Products Industries Spectro grade acetone has also been used. Acetone from other sources may cause high reagent blanks because of impurities similar to glyoxal. It may be purified in the same way as the alcohol. The acetone used for developing solutions in the final chromatographic purification of glyoxal 2,4-dinitrophenylosazone need not be highly purified, however.

20% Nitroethane in benzene. Dilute 20 ml. of nitroethane to 100 ml. with benzene. Nitroethane (Commercial Solvents Corp.) has been successfully used without purification; but if purification is necessary, reflux with 2,4-dinitrophenylhydrazine and a small amount of sulfuric acid, wash with water, dry, and distill. Collect the fraction boiling at 114° C.

Alumina, Woelm, acid (anionotropic) and neutral. Obtained from Alupharm Chemicals, 322 Lafayette St., New Orleans, La. To the alumina add 2% of its weight of distilled water and tumble for several hours. Store in and dispense from containers carefully protected from atmospheric water vapor.

Tetramethylammonium hydroxide (TMAH), 10% aqueous solution (Distillation Products Industries).

Hexane. Skellysolve B, Skelly Oil Co., is satisfactory.

Chromatographic tubes, No. 1, Scientific Glass Apparatus Co., Bloomfield, N. J., catalog No. J-1663.

Fisher Filtrator, Fisher Scientific Co.

Chromatographic rack (Figure 2) or similar device. The apparatus consists of an aluminum base plate fitted with support rods, a vacuum manifold (each outlet having a three-way valve), and a pressure manifold fed by a Conoflow regulator.

Preparation of Standard Curve. Standard curves may be prepared using either pure Delnav or technical Delnav. When the latter is used, interfering glyoxal precursors are first removed by use of alumina, the purified material made into solution without weighing, and the concentration of the solution expressed in terms of the weight of technical Delnav originally taken. Aliquots of the standard solution are taken to cover the range 10 to 170 γ of technical Delnav (7 to 120 γ of pure Delnav), performing the analyses as described under Procedure, and making the solution for color measurement to a volume of 10 ml. A second curve covering the range 5 to 85 γ of technical Delnav (3 to 60 γ of pure Delnav) may also be prepared, using a 5-ml. final volume for color measurement.

Plot absorbance at 614 mμ against micrograms of Delnav. The points so determined should be on a straight line, but the line probably will not pass through the origin. The absorbance indicated at the zero intercept is that of the reagent blank. Because the chromatographic purification of the glyoxal derivative involves a visual determination of when to start and stop collecting the desired eluate, the reagent blank determined by extrapolation to zero concentration will be more reliable than that determined by running actual reagent blanks, where it is difficult to determine the exact amount of eluate to collect.

If pure Delnav is used for the standard solution, a hexane solution containing about 30 γ per ml. is convenient. When technical Delnav is used for the standard solution, prepare a 10-cm. column of Woelm acid alumina (2% added water) in a No. 1 chromatographic tube. From a weighed vial of technical Delnav, transfer with a dropper about 100 mg. (determine weight to ±0.1 mg.) directly on the alumina. Pass through the column 25 ml. of hexane, discarding this eluate. Elute with 30 ml. of benzene, collecting the eluate. Evaporate essentially all of the benzene, transfer the residue to a volumetric flask, and dilute to volume with hexane. This solution may be used directly if it is sufficiently dilute. Otherwise, dilute an aliquot to provide a final concentration in the range of 30 to 50 γ of technical Delnav per ml.

Procedure. Pipet into a 12-ml. graduated conical centrifuge tube the hexane solution of Delnav. Evaporate the hexane by heating the tube in a 60° C. water bath and blowing a stream of air into the tube. Cool the tube, wash down the sides with a small volume of hexane (not more than 0.3 ml.), and again evaporate the hexane. Add 0.3 ml. of 0.2*M* alcoholic mercuric chloride solution and 0.3 ml. of 0.08*M* 2,4-dinitrophenylhydrazine reagent. Heat the tube in a thermostatically controlled 80° C. bath for 30 minutes. Cool to room temperature and add 0.2 ml. of acetone to consume excess 2,4-dinitrophenylhydrazine.

Wash the reaction mixture into a 30-ml. beaker with 20% nitroethane in benzene, heating the final rinses to ensure the complete solution of the glyoxal 2,4-dinitrophenylosazone. Prepare a 10-cm. column of Woelm acid alumina (2% added water) in a No. 1 chromatographic tube that is fitted to a Fisher Filtrator. Decant the nitroethane-benzene solution from the small amount of water in the 30-ml. beaker, and rinse the column with sufficient

nitroethane-benzene just to remove the yellow zones of hydrazones and glyoxal 2,4-dinitrophenylosazone. A band of tars and other undesired reaction products may follow the yellow band on the column; do not elute this material because it is difficultly soluble in benzene and complicates the succeeding chromatographic purification of the glyoxal derivative. Evaporate the eluate by heating it on the steam bath under a stream of air.

Prepare a 35-cm. column of Woelm acid alumina (2% added water) in a 6-mm. outside diameter glass tube by first inserting a pad of glass wool and admitting about 5 to 10 cm. of alumina. Apply vacuum to draw in enough alumina to give a column of about 35 cm. when it is packed by tapping the sides of the tube. Attach a small funnel to the top of the tube with rubber tubing. Dissolve the residue from the nitroethane solution by boiling with a few milliliters of benzene. Add the yellow benzene solution to the alumina column with vacuum applied and follow with benzene rinses that were also boiled. Dissolving all of the glyoxal derivative is sometimes difficult, and thorough heating of the benzene rinses is essential to achieve complete recovery. Check the completeness of removal of the glyoxal derivative by adding a few drops of *N,N*-dimethylformamide and a drop of tetramethylammonium hydroxide to the beaker after the final rinse. A blue color will show the presence of glyoxal 2,4-dinitrophenylosazone.

After the last benzene rinse has been drawn into the column, attach a 50-ml. hypodermic syringe (without plunger) and needle (No. 20, 1-inch) to the column by means of a number 12½ D rubber sleeve stopper (serum stopper). To the syringe add about 35 ml. of 2.5% acetone in benzene and develop the column. Solvent flow will be speeded if pressure (about 5 p.s.i.) is applied to the syringe and vacuum to the lower end of the chromatographic tube. The first compounds to be eluted will be the 2,4-dinitrophenylhydrazones, while the glyoxal 2,4-dinitrophenylosazone will follow, moving at a slower rate. The glyoxal derivative may be eluted with 5% acetone in benzene after it is sufficiently separated from the other compounds on the column. Collect the eluate containing the glyoxal derivative in a 30-ml. beaker and evaporate to dryness on a steam bath under an air stream.

Neutral alumina may also be used for the purification of the glyoxal 2,4-dinitrophenylosazone, and the operation can be done more rapidly with this adsorbent. The benzene solution is drawn into the column as described for the acid alumina, and the column developed with about 10 ml. of 5% acetone in benzene to remove hydrazones, and then with about 5 ml. of 50% acetone in benzene to remove the glyoxal derivative quickly. The choice of acid or neutral alumina is based on performance under the conditions encountered. When adequate purification is achieved with the neutral alumina, its use is recommended.

Add to the dry residue of glyoxal 2,4-dinitrophenylosazone about 2 ml. of *N,N*-dimethylformamide, 1 drop of tetramethylammonium hydroxide, and 1 drop of water. Heat for 10 seconds on a steam bath and transfer the solution to a 5- or 10-ml. volumetric flask. Rinse with *N,N*-dimethylformamide and make to volume with *N,N*-dimethylformamide after adding additional tetramethylammonium hydroxide and water to make the total two drops of tetramethylammonium hydroxide and two drops of water for each 5 ml. of final solution. Moderate excesses of these reagents are of no consequence. Any cloudiness of the solution can be cleared with a few additional drops of water. Cool the solution to room temperature.

Read the absorbance of the solution at 614 mμ using 1-cm. cells with the Beckman Model B or similar spectrophotometer, and using distilled water for reference.

Calculation. The absorbance of the solution is related to the amount of Delnav by use of standard absorbance–concentration curves prepared as described under Preparation of Standard Curves:

$$\frac{\text{Micrograms of Delnav}}{\text{grams of sample}} = \text{p.p.m. of Delnav}$$

Extraction of Delnav from Crops

The sample taken for analysis should be truly representative of the entire mass of material made available for the residue determination. By plugging, chopping, or grinding, the particle size should be reduced to the point where blending and subsampling will yield a final quantity of material that is made up of small contributions from each part of the entire sample. Hexane or 2 to 1 hexane–isopropyl alcohol is recommended for the extraction of Delnav from the sample. Skellysolve B (60° to 70° C. boiling range, Skelly Oil Co.) is a satisfactory grade of hexane.

The sample may be minced in the presence of the solvent and tumbled, or if the particle size is already small, the sample may be simply tumbled with the solvent. In general, at least 1 hour of tumbling should be performed, using an amount of solvent such that 1 or 2 ml. of final hexane solution will represent 1 gram of sample. After tumbling, decant the hexane extract into a separatory funnel and wash with water several times; dry the extract over a drying agent such as anhydrous magnesium sulfate or sodium sulfate. If 2 to 1 hexane–isopropyl alcohol has been used, wash each liter of extract with 400-, 200-, and 200-ml. portions of water to ensure complete removal of the isopropyl alcohol, and dry the hexane extract.

Isolation of Delnav from Hexane Extract and Cleanup to Remove Extraneous Materials

In many cases, Delnav can be adsorbed on alumina from hexane solution. Many of the waxes and other materials are not adsorbed, and are readily eluted with hexane. Delnav can be eluted from the alumina with benzene, whereas many pigments will be retained. Final cleanup to remove nearly all remaining extraneous material can be conveniently done by partition chromatography with a solvent pair such as acetonitrile-hexane. Acetonitrile (Union Carbide Chemicals Co.) has been used successfully without purification. In the case of oils or fats, a preliminary separatory funnel partitioning may be necessary prior to the alumina isolation procedure.

Select an aliquot of the dried hexane extract estimated to contain 10 to 100 γ of Delnav. If it is more than 50 ml., reduce its volume by evaporative concentration, preferably in a Kuderna-Danish type evaporator. If necessary, redry the concentrated solution over magnesium sulfate or sodium sulfate.

Prepare a 10-cm. column of Woelm acid alumina (2% added water; refer to Apparatus and Reagents under Cleavage-Hydrolysis Procedure) in a No. 1 chromatographic tube. Protect the upper surface of the adsorbent with a disk of filter paper. Connect to the top of the tube a 50-ml. hypodermic syringe (without plunger) and needle by means of a number 12½ D rubber sleeve stopper (serum stopper). Transfer the hexane extract into the hypodermic syringe, and draw the extract into the chromatographic tube by applying vacuum to the bottom of the tube. Follow with two 10-ml. rinses of hexane, and discard the hexane eluate. Add 30 ml. of benzene to the hypodermic syringe and elute the column, collecting the eluate. Evaporate the benzene under an air stream on a steam bath, taking care not to heat beyond the point of benzene removal.

Prepare a partition column by packing 7.0 grams of Celite 545 (Johns-Manville Co.) to a height of 23.5 cm. in a 10-mm. (inside diameter) chromatographic tube (A. H. Thomas Co., Catalog No. 3199-R is convenient). This is accomplished by filling the column a little more than half full under vacuum and tapping the side of the tube with a wooden rod until the Celite is well packed. Add the remaining Celite and repeat the tapping. Disconnect the vacuum, smooth the surface of the Celite, and insert a disk of filter paper to prevent the Celite from being disturbed when liquids are added. Pipet 4.65 ± 0.05 ml. of acetonitrile (equilibrated

with hexane) into the chromatographic tube, and apply pressure (about 2 p.s.i.) to move the acetonitrile into the column. Then add hexane (equilibrated with acetonitrile) and again apply pressure to wet the entire column. Release the pressure when the solvent level is a few millimeters above the filter paper disk.

Dissolve the residue from the evaporation of the benzene eluate in a few milliliters of hexane (equilibrated with acetonitrile) and add it to the chromatographic tube. Apply pressure to move the sample solution into the column, but do not allow the level of liquid in the column to drop below the filter paper disk. Follow with rinsings, keeping the total volume of sample solution and rinsings to no more than 5 ml. Develop the column with hexane (equilibrated with acetonitrile). Collect the eluate, starting with the first amount of sample entered into the column. Discard the first 50 ml. and retain the next 100 ml. The 100-ml. fraction contains the Delnav.

Concentrate the eluate to about 2 ml. by evaporating it under an air stream on a steam bath and transfer it to a 12-ml. conical centrifuge tube. Immerse the tube in a 60° C. water bath and continue the evaporation under an air stream. When the hexane is evaporated, cool the tube and wash down the sides with hexane to concentrate all of the residue below the 0.4-ml. mark. Repeat the washing and evaporation if necessary. A medicine dropper drawn out to a capillary will allow a thorough rinsing of the sides of a cool centrifuge tube with no more than 0.3 ml. of hexane.

At this point, the Delnav has been freed from the major part of the waxes, pigments, and other extraneous materials which were extracted from the sample. The next step is the actual determination of the Delnav by the colorimetric cleavage-hydrolysis procedure.

Experimental Work

Glyoxal 2,4-Dinitrophenylosazone, Formation and Recovery. A sample of highly purified 2,3-dibromo-*p*-dioxane was used as a source of glyoxal to establish the accuracy and precision of the glyoxal 2,4-dinitrophenylosazone (glyoxal DNO) formation and recovery. Because Delnav and 2,3-dichloro-*p*-dioxane are essentially insoluble in water, the use of an alcohol-water system to promote a more nearly homogeneous system for cleavage-hydrolysis was established. Alcoholysis of 2,3-dichloro-*p*-dioxane to yield 2,3-diethoxy-*p*-dioxane could be a reaction competitive with hydrolysis and therefore the rate of hydrolysis of the acetal was also checked.

Hydrolysis of 2,3-Dibromo-*p*-dioxane. Aliquots containing 69.46 mg. of 2,3-dibromo-*p*-dioxane were refluxed for 15 minutes with 15 ml. of 0.08*M* 2,4-dinitrophenylhydrazine (DN) in 4*M* perchloric acid and 10 ml. of alcohol. Precipitates were washed with water, 1 to 1 water-alcohol, alcohol, carbon disulfide, and alcohol, then dried for 15 minutes at 105° C. in a forced-draft oven. Found, 117.3, 117.4, 117.6, and 117.9 mg. Calculated, 118.1 mg.

Hydrolysis of 2,3-Diethoxy-*p*-dioxane. 2,3 - Diethoxy - *p* - dioxane was refluxed with hydrochloric and perchloric acid solutions of dinitrophenylhydrazine in aqueous alcohol for 15 and 60 minutes. Solutions were 45% alcohol, 1.6*N* hydrochloric acid or 2.4*N* perchloric acid, and 0.01*M* 2,3-diethoxy-*p*-dioxane. Formation of glyoxal 2,4-dinitrophenylosazone was at least 95% complete within 15 minutes—using the perchloric acid reagent—while the hydrochloric acid reagent gave less than 90% hydrolysis in 60 minutes. Mercuric chloride at a concentration of 0.1*M* had no effect on any of the results.

Hydrolysis of 2,3-Dichloro-*p*-dioxane. Aqueous alcohol (50% v./v.) solutions of 2,3-dichloro-*p*-dioxane ($3 \times 10^{-3}M$) yielded 99% of the calculated amount of glyoxal 2,4-dinitrophenylosazone after a 5-minute reflux period. At room temperature, the glyoxal 2,4-dinitrophenylosazone found after 16 hours was 97% of the calculated amount. Mercuric chloride (0.1*M*) had no effect at either room or reflux temperature.

Melting Point of Glyoxal 2,4-Dinitrophenylosazone. Melting point of the glyoxal derivative prepared by hydrolysis of 2,3-dichloro-*p*-dioxane was 323° C.; after one recrystallization from nitromethane, 326° C.

Cleavage of Delnav with Mercuric Chloride. The addition of mercuric chloride to an alcohol solution of Delnav causes the immediate formation of a precipitate. The filtrate will yield a precipitate of glyoxal 2,4-dinitrophenylosazone immediately upon the addition of 2,4-dinitrophenylhydrazine reagent. The identity of the precipitate formed by the addition of mercuric chloride to Delnav has not been established. X-ray diffraction patterns showed that it is neither mercuric nor mercurous chloride. It is completely soluble only in reagents such as aqua regia and bromine water, and may be a decomposition product of *S*-chloromercuric *O,O*-diethyl phosphorodithioate, because it continues to form for some time after cleavage of the Delnav is complete. Positive qualitative tests for chlorine, mercury, phosphorus, and sulfur were obtained when the material was decomposed by sodium fusion.

Even with 20-fold excess of mercuric chloride, a reflux period of more than 1 hour is necessary to precipitate all of the mercury compound if it is to be removed prior to formation of the glyoxal derivative. An alternative approach was to find a means of ridding the glyoxal derivative of the mercury precipitate so that the glyoxal can be precipitated as it forms. Only one solvent, dimethylformamide, was found in which glyoxal 2,4-dinitrophenylosazone is sufficiently soluble to be useful in selectively dissolving it away from the mercury precipitate.

The mercury precipitate is affected slightly by the *N,N*-dimethylformamide, and when more than 40 mg. is present there may be sufficient solubility to cause an error of several per cent in determining the weight of the glyoxal derivative. When the cleavage, hydrolysis, and derivative formation were performed in one operation and the glyoxal 2,4-dinitrophenylosazone was determined by dissolving it in hot *N,N*-dimethylformamide, reflux periods of 15, 30, and 60 minutes gave identical (±0.2%) results. When the reflux period was extended to 2 and 4 hours, results were high by 1.5 and 4.4%. Weights of the mercury precipitates ranged from 23 to 43 mg. for reflux periods up to 1 hour and increased to 80 mg. for a 4-hour reflux.

Effect of Sample Size. A series of determinations was made over the range of 4 to 110 mg. of Delnav, using twofold and 20-fold excesses of mercuric chloride. The weights of the glyoxal 2,4-dinitrophenylosazone were determined by the difference in weight of the total precipitate and that remaining after leaching with hot *N,N*-dimethylformamide. No difference in results was found with this wide variation in reagent concentration. The weight of the glyoxal 2,4-dinitrophenylosazone was directly proportional to the amount of sample but the weights of the mercury precipitates were an inadequate measure of the amount of sample taken.

Acid Hydrolysis of Delnav. Delnav is hydrolyzed in aqueous acid solutions to yield glyoxal, but the reaction is much slower than the cleavage with mercuric chloride. When equal volumes of 4*N* perchloric acid and $6 \times 10^{-3}M$ alcohol solution of Delnav were refluxed, only half of the Delnav had reacted to yield glyoxal after 1 hour.

Removal of Coprecipitants and Contaminants from Glyoxal 2,4-Dinitrophenylosazone. Mercury Precipitate. The mercury precipitate, because of its slight solubility in hot *N,N*-dimethylformamide and the uncertainty regarding the constancy of its composition, is an undesirable material to include in the measurement of the glyoxal 2,4-dinitrophenylosazone. The mercury precipitate is soluble in bromine water and aqua regia and is decomposed to form mercuric sulfide by sodium sulfide or ammonium polysulfide. The use of sulfide reagents (which also dissolve the glyoxal derivative) was not successful, how-

ever, because the mercuric sulfide formed was frequently colloidal, could not be filtered, and was adsorbed on the glyoxal 2,4-dinitrophenylosazone when the latter was reprecipitated. When thioacetamide was used to precipitate mercuric sulfide, the particles were larger, but the time required was excessive.

The melting point of glyoxal 2,4-dinitrophenylosazone treated with bromine water was 320° C., indicating little if any effect. A more convenient reagent, a solution of potassium iodide in hydrochloric acid (15 grams of potassium iodide, 10 ml. of concentrated hydrochloric acid, and 85 ml. of water), was also shown to have no effect on the glyoxal 2,4-dinitrophenylosazone.

Other Materials Extracted with Carbon Disulfide. The odor of glyoxal 2,4-dinitrophenylosazone from the cleavage-hydrolysis of technical Delnav suggested that some sulfur-containing material was still present, despite washing with potassium iodide–hydrochloric acid reagent, bromine water, and alcohol. Carbon disulfide was a good solvent not only for the odorous materials but for monocarbonyl 2,4-dinitrophenylhydrazones as well.

Extraction with carbon disulfide of glyoxal 2,4-dinitrophenylosazone prepared from technical Delnav removed about 5% of the precipitate. Similar extraction of glyoxal 2,4-dinitrophenylosazone prepared by hydrolysis of 2,3-diethoxy-*p*-dioxane removed about 2% of the material. Colorimetric determination of the glyoxal 2,4-dinitrophenylosazone separated chromatographically from 3.5 mg. of the latter extract showed it to contain only 29 γ of glyoxal 2,4-dinitrophenylosazone.

Removal of Other Glyoxal Precursors from Technical Delnav. Delnav can be adsorbed on alumina from hexane solution, but is readily eluted by benzene, whereas other glyoxal precursors present in technical Delnav are retained by the alumina. The moisture content of the alumina is critical in this application, but the use of a standardized adsorbent such as the Woelm product makes the adsorbent cleanup a practical operation. A series of fractions obtained by alumina treatment of samples of technical Delnav was analyzed by the gravimetric cleavage-hydrolysis procedure and gave results ranging from 94 to 103% Delnav (Table I). When alumina of higher water content was used, values as high as 122% were obtained, indicating the elution of other glyoxal precursors along with the Delnav. Woelm alumina to which 2% of its weight in water is added was in the most useful range of activity.

Analysis of Purified Delnav and Effect of Reflux Period. A sample of Delnav isolated from technical Delnav by partition chromatography was analyzed using a 15-minute reflux period and equal volumes of 0.2*M* alcoholic mercuric chloride and 0.08*M* 2,4-dinitrophenylhydrazine in 4*N* perchloric acid. The filtrates were collected and refluxed for an additional 45 and 60 minutes to see if additional amounts of glyoxal 2,4-dinitrophenylosazone were formed. The results show the reactions to be complete within 15 minutes and the amount of glyoxal 2,4-dinitrophenylosazone recovered to be about 98% of the calculated yield. Samples of 57.1 and 25.6 mg. of Delnav gave 51.5 and 22.8 mg. of glyoxal 2,4-dinitrophenylosazone, respectively, and no additional precipitate of this derivative was formed by further refluxing of the filtrates. These amounts of glyoxal 2,4-dinitrophenylosazone are 98.4 and 97.2% of the calculated yield.

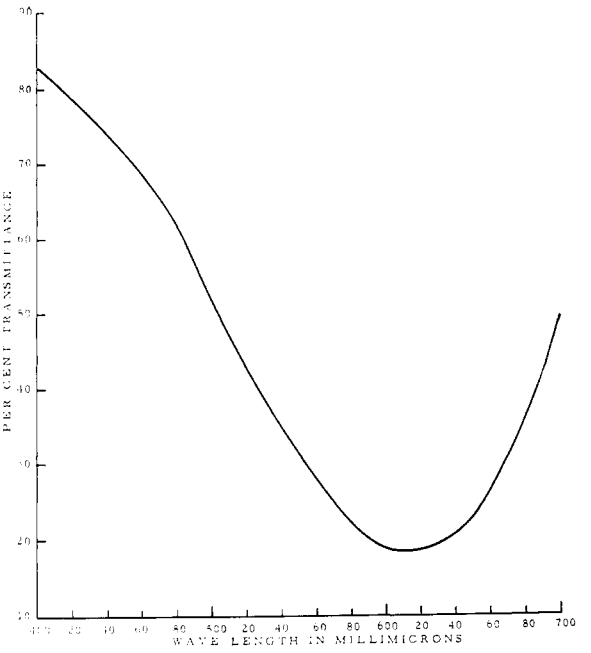


Figure 1. Transmittance–wave length curve for glyoxal 2,4-dinitrophenylosazone in dimethylformamide containing aqueous tetramethylammonium hydroxide

Colorimetric Method

Spectrophotometric Characteristics of Glyoxal 2,4-Dinitrophenylosazone. The determination of carbonyl compounds by measuring the intense colors formed when their 2,4-dinitrophenylhydrazones are made basic has been widely used (*10*, *12*). The monocarbonyl derivatives are generally red, whereas the dicarbonyl derivatives are usually blue. Dimethylformamide was chosen as a solvent because it readily dissolves glyoxal 2,4-dinitrophenylosazone, is nonvolatile, and is miscible with the aqueous tetramethylammonium hydroxide used to make the solution basic. The latter reagent was selected in preference to the more conventional sodium hydroxide or sodium methylate because it is a strong base but is little affected by carbon dioxide and yields no insoluble sodium carbonate upon exposure to air.

A transmittance–wave length curve for a basic dimethylformamide solution of glyoxal 2,4-dinitrophenylosazone is shown in Figure 1, and indicates that it is well suited for a colorimetric method. The absorbance maximum is at 614 mμ, and the absorbance-concentration curve was linear up to an absorbance of 2. The stability of the color with time is excellent. Solutions containing 1 to 4 γ of glyoxal 2,4-dinitrophenylosazone per ml. had unchanged absorbance after 72 hours at room temperature.

The following values were determined for constants that are useful in defining the sensitivity of a colorimetric method (*4*): absorptivity, 176 liters per gram cm.; molar absorptivity, 73.5 × 10^3 liters per mole cm.; absorbance concentration (concentration in micrograms per milliliter required to give an absorbance of 1.00, using 1-cm. cell), 5.7 γ per ml. Measurements were made at room temperature with a Beckman Model B spectrophotometer and 1-cm. cells. Reference solution was *N,N*-dimethylformamide to which tetramethylammonium hydroxide and water had been added in the amounts specified in the detailed analytical procedure. Es-

Table I. Analysis of Alumina-Treated Technical Delnav

Sample No.	Sample, Mg.	Glyoxal DNO, Mg.	Calcd. as % Delnav
A-1	69.2	61.9	97.4
2	50.2	45.1	98.4
3	46.3	42.4	99.6
B-2	40.3	34.7	93.9
4	45.9	42.7	101.5
6	50.2	45.4	98.6
8	42.2	39.8	102.8
10	44.8	40.7	99.0
12	45.5	40.9	98.0
14	46.4	43.3	101.8

Table II. Effect of Heating Period on Microgram Scale Cleavage-Hydrolysis of Delnav

Al_2O_3-Treated Delnav, γ	Heating at 80° C., Min.	Delnav Found, γ
62	5	56
62	15	62
62	30	64
62	30	62
62	30	63
62	60	64

sentially no difference was found between absorbance of this solution and that of distilled water, however.

The sensitivity is high for, according to Mellon (*8*), the highest molar absorptivities previously encountered in colorimetry were near 35,000, which is only half that determined for glyoxal 2,4-dinitrophenylosazone. A recent publication by Banks, Vaughn, and Marshall (*1*) on the spectrophotometric determination of glyoxal 2,4-dinitrophenylosazone in alkaline acetone gives the absorbance maximum as 600 mμ, but fails to give data for sensitivity measurements.

Cleavage-Hydrolysis of Delnav on Microgram Scale. The cleavage-hydrolysis reaction of Delnav operates satisfactorily on a microgram scale. Small amounts of glyoxal 2,4-dinitrophenylosazone could not, however, be satisfactorily recovered by the method of Neuberg and Strauss (*12*), wherein kaolin or other solids are used for the adsorption-precipitation of the glyoxal derivative.

Solvent System. An aqueous system was found entirely satisfactory when analyses are made with only Delnav present—for example, when running standard curves. If waxes and other crop extractives are present, however, the use of a solvent such as alcohol in the reaction mixture is desirable to promote a more nearly homogeneous solution.

Reaction Time and Temperature. At a temperature of 80° C., a reaction period of 15 minutes was adequate using 0.3-ml. volumes of 0.08*M* 2,4-dinitrophenylhydrazine in 4*N* perchloric acid and 0.2*M* alcoholic-mercuric chloride. Thirty minutes was chosen for the general procedure to provide a safety factor that might be necessary in the presence of waxes and other crop extractives. Data regarding the analysis of a standard solution of Delnav using reaction periods ranging from 5 to 60 minutes are given in Table II.

Isolation and Purification of Glyoxal 2,4-Dinitrophenylosazone. To make a satisfactory colorimetric determination of the glyoxal 2,4-dinitrophenylosazone formed in the reaction mixture, other colored materials must be removed.

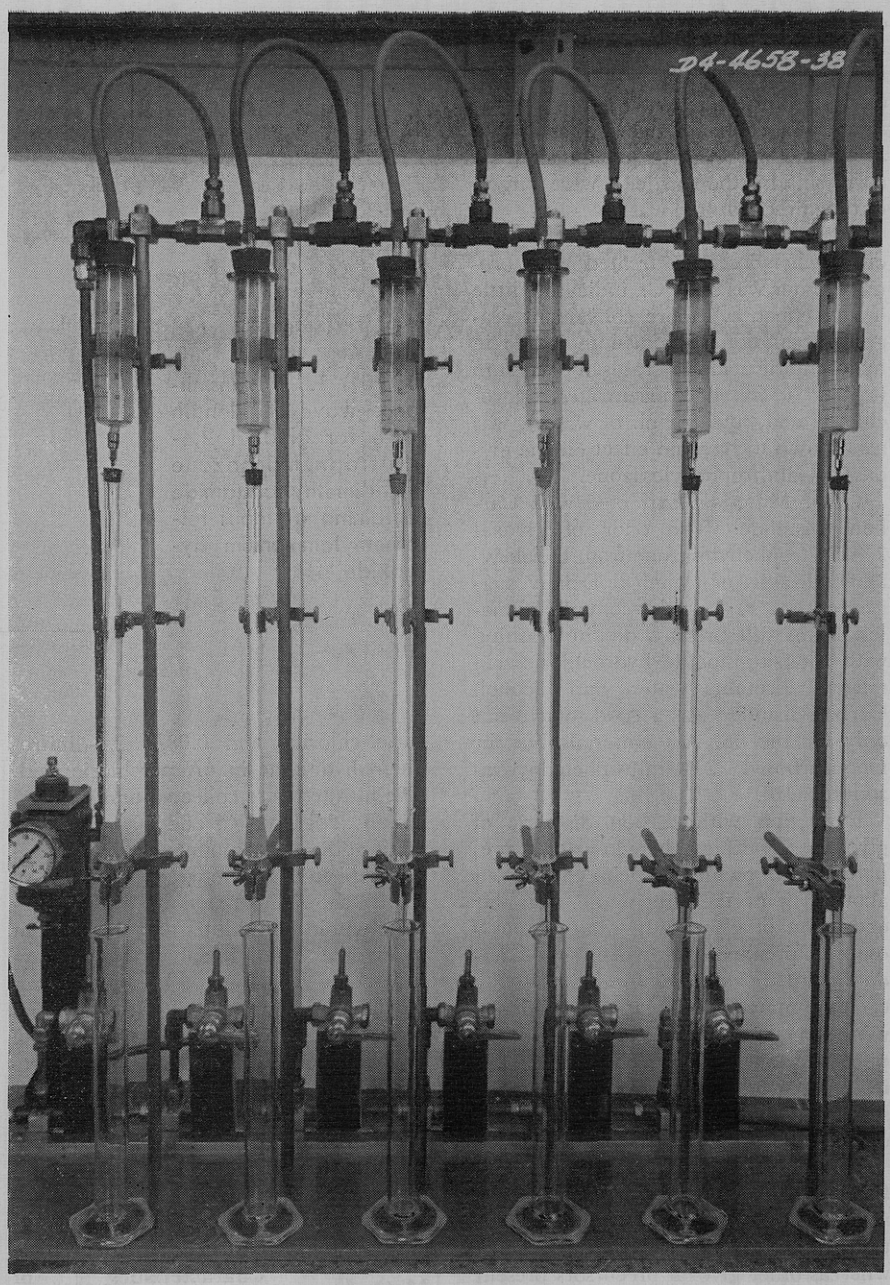


Figure 2. Chromatographic rack set up for partition chromatography in cleanup of crop extracts

This apparatus for holding chromatographic tubes and providing vacuum and pressure manifolds has general utility in partition chromatography. It was used for the adsorption of Delnav on alumina and for the purification of glyoxal 2,4-dinitrophenylosazone (colorimetric procedure), with a simple change of tubes and receptacles

These include excess reagent, other 2,4-dinitrophenylhydrazones, pigments, and other crop extractives. Chromatographic separation seemed the most likely method for removal of these unwanted compounds (*7*, *10*, *14*), and silicic acid and alumina were tried as adsorbents. Satisfactory separation of glyoxal 2,4-dinitrophenylosazone can be achieved using either of these, but alumina was finally chosen because of good solvent flow rate through it and because it can be obtained commercially in a fairly consistent degree of activity.

The hydration of the alumina is highly critical, as is the basicity or acidity. Woelm alumina to which 2% of its weight in water is added was of greatest use for this separation, and coincides with the type of alumina used for cleanup of technical Delnav. Glyoxal 2,4-dinitrophenylosazone on acid alumina (2% added water) is developed slowly with 2.5% acetone in benzene, but is nearly immobile on neutral alumina when this solvent is used.

The entire reaction mixture is dissolved in nitroethane or benzene-nitroethane. This solution is dried by filtering it through alumina. This also re-

moves certain tars and other undesired reaction products. After evaporation of the nitroethane the residue can be dissolved in benzene and the glyoxal separated by chromatography on alumina. The problem of excess reagent reacting with impurities in benzene and nitroethane to yield interfering substances was solved by the addition of acetone to consume excess reagent. The resulting hydrazone is easily separated from the glyoxal 2,4-dinitrophenylosazone.

Oxidation of Ethylene Glycol. Hydrolysis of 2,3-dichloro-*p*-dioxane yields both ethylene glycol and glyoxal. Oxidation of the ethylene glycol to glycoaldehyde or glyoxal was tried, with emphasis on the use of persulfate ion with ferrous ion (*9*). Not only ethylene glycol is oxidized, but impurities in materials such as reagent grade ether, reagent grade aqueous formaldehyde, acetone, and alcohol, are also oxidized to yield glycoaldehyde, glyoxal, or very similar products. The complications created by oxidation of solvent impurities made this oxidation undesirable despite doubling of the yield of glyoxal.

Precision, Accuracy, and Sensitivity of Colorimetric Method. A solution of alumina-purified Delnav containing 31.0 γ per ml. in hexane was used. Six replicate determinations of each level of 9.3 and 62 γ of Delnav were made (Table III). The standard deviation at the 62-γ level is 0.026 absorbance unit, equivalent to 1.6 γ of Delnav. At the 9.3-γ level, the standard deviation is 0.031 absorbance unit, equivalent to 0.95 γ of Delnav.

The sensitivity constants calculated from these data are compared in Table IV with those calculated from the measurements made on glyoxal 2,4-dinitrophenylosazone solutions. Agreement is within 3%, and this is regarded as adequate confirmation of the quantitative conversion, on a microgram scale, of Delnav to glyoxal and recovery of the glyoxal derivative.

Range of Colorimetric Method. The method was demonstrated to be linear over a range of 8 to 500 γ of technical Delnav. For larger amounts of Delnav, the final solutions were diluted to obtain absorbances within the range of the spectrophotometer. It is unnecessary to limit closely the amount of Delnav in the sample analyzed (Table V).

Table III. Precision and Sensitivity of Colorimetric Cleavage-Hydrolysis Procedure

Purified Delnav, γ	DMF Solution, Ml.	Absorbance at 614 Mμ, 1-Cm. Cells
62	10	1.089, 1.080, 1.019, 1.065, 1.090, 1.075 Av. 1.070 Standard deviation = 0.026 ≃ 1.62 γ Delnav
9.3	5	0.375, 0.438, 0.375, 0.410, 0.374, 0.400 Av. 0.393 Standard deviation = 0.031 ≃ 0.95 γ Delnav

Table IV. Calculated and Observed Sensitivity Constants for Colorimetric Method

Constant	Found for Delnav	Calcd. from Glyoxal DNO Data
Absorptivity, liters per gram cm.	166	161
Molar absorptivity, liters per mole cm.	75.5×10^3	73.5×10^3
Absorbance concentration, γ/ml.	6.03	6.20

Discussion

Use of perchloric acid solution of 2,4-dinitrophenylhydrazine as suggested by Neuberg, Grauer, and Pisha (*11*) is a considerable improvement over the conventional hydrochloric or sulfuric acid solutions. The gravimetric cleavage-hydrolysis procedure has been applied to the analysis of technical Delnav and various formulations such as emulsion concentrate, dust, and wettable powder.

The colorimetric modification of the method has been demonstrated to be practical for residue analysis. More than 500 samples from crops such as grapes, apples, oranges, lemons, cottonseed oil, and milk have been analyzed. In all cases, combinations of adsorption on alumina and partition cleanup have been adequate for the determination of Delnav residues. In the case of oils and fats, preliminary separatory funnel partitioning between solvents such as acetonitrile and hexane have been required before alumina treatment.

Use of the apparatus shown in Figure 2 has made practical the routine application of the various chromatographic procedures to residue analyses.

Table V. Application of Colorimetric Method over Range 8 to 500 γ

Delnav, γ	DMF Solution, Ml	Absorbance at 614 mμ, 1-Cm. Cells: Observed	Corr. for reagent blank	Calcd. to 5-ml. vol.
8.1	5	0.232	0.188	0.188
16.2	5	0.402	0.358	0.358
32.4	5	0.760	0.716	0.716
64.8	10	0.700	0.678	1.356
122.0	50	0.261	0.256	2.56
243.0	50	0.510	0.505	5.05
486.0	100	0.500	0.498	9.96
0.0	5	0.040	...	...
0.0	5	0.049	...	...

Acknowledgment

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